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Intracellular targeting and import of an F_1-ATPase β-subunit–β-galactosidase hybrid protein into yeast mitochondria

(gene fusion/mitochondrial biogenesis/protein import/targeting signals)

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ABSTRACT **The gene coding for the yeast mitochondrial F_1-ATPase β subunit (*ATP2*) has been fused to the *Escherichia coli lacZ* gene. The chimeric *ATP2–lacZ* gene codes for a hybrid protein consisting of some 350 amino acids of the F_1-ATPase β subunit at its amino terminus and a large enzymatically active portion of the *lacZ* gene product, β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23), at its carboxyl terminus. The β-subunit–β-galactosidase hybrid protein is expressed in both *E. coli* and yeast. In yeast, this hybrid molecule is targeted to the mitochondrion and is protected in isolated mitochondria from added protease under conditions in which an outer membrane enzymatic marker is digested. Yeast cells carrying the *ATP2–lacZ* gene fusion on plasmid pβZ1 are unable to grow on a nonfermentable carbon source. Upon loss of the pβZ1 plasmid, growth of the cured host strain on the nonfermentable substrate is restored. In the presence of the β-subunit–β-galactosidase hybrid protein, the energy-transducing capacity of the mitochondrial membrane as measured by the $^{32}P_i$–ATP exchange reaction is only 9% of that measured in the absence of the gene fusion product. The results indicate that it is the presence of the β-subunit–β-galactosidase hybrid protein within mitochondria that interferes with function(s) essential for respiratory growth. These observations open up the prospect of genetic characterization of the signals and cellular machinery responsible for mitochondrial protein delivery.**

Most mitochondrial proteins are encoded by nuclear genes (1). The proteins are synthesized in the cytoplasm prior to being directed to their specific submitochondrial destinations; the mitochondrial matrix, the inner membrane, the outer membrane, or the intermembrane space (2, 3). Specific targeting of these proteins to a unique submitochondrial compartment is presumed to be directed by information contained within each polypeptide. Indeed, several mitochondrial proteins are made initially as larger precursors that possess transient "pre sequences" that are required for import of these proteins into mitochondria (4). We are interested in understanding the nature of the information responsible for this delivery process.

One approach to addressing this question involves the use of gene fusions. By fusing various portions of a gene coding for an imported mitochondrial protein to a gene such as *lacZ*, which codes for the easily detected enzyme β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23), it should be possible to define that portion of the gene which is responsible for delivery of its product to the mitochondrion. A similar approach already has been employed successfully to study various features of the molecular events in the process of protein secretion in *Escherichia coli* (5–9).

In this study, we have chosen to analyze the delivery of the β-subunit protein of the mitochondrial F_1-ATPase. This subunit of the ATPase complex, as well as six others, is known to be coded for by a nuclear gene (10, 11). The β subunit is synthesized initially as a 56-kilodalton (kDa) precursor with an amino-terminal pre sequence of approximately 2 kDa (4). *In vitro* studies have demonstrated that the pre segment is necessary for import of the β subunit to its final destination in the mitochondrial matrix space (12, 13). We recently have cloned the gene coding for the β-subunit protein, *ATP2*, by genetic complementation of a yeast *atp2* mutant (14, 15). The availability of such a clone and the existing data regarding the import and localization of the β-subunit protein make it a good candidate for gene fusion study.

We report here the construction and characterization of a gene fusion between a portion of the *ATP2* gene, encoding 350 amino-terminal residues of the β-subunit protein (ref. 15; unpublished data) and a portion of *lacZ* encoding a large carboxyl-terminal active fragment of β-galactosidase.

MATERIALS AND METHODS

Strains and Media. *E. coli* strain MC1061 [F⁻ *araD139* Δ(*lacIPOZYA*)X74 Δ(*araABOIC–leu*)7697 *galK hsdR⁻ hsdM⁺ rpsL*] was provided by M. Casadaban. Biochemical studies were done with a good transforming strain of *Saccharomyces cerevisiae*, SEY2101 (*MATα leu2-3 leu2-112 ura3-52 his4-519 suc2-Δ9*) (16).

E. coli were grown in LB medium (ref. 17, p. 433) and ampicillin was added when required to a final concentration of 100 μg/ml. Bacterial indicator plates containing 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) were prepared as described (ref. 17, p. 54). Yeast were grown in YEPD, YEPG, or 2× synthetic minimal medium supplemented with the indicated sugar (ref. 18, pp. 61–63). Yeast X-Gal indicator plates were prepared essentially as described by Rose *et al.* (9).

Transformations and Plasmid Analysis. Transformation of *E. coli* (ref. 19, pp. 250–251) and yeast (ref. 20) with plasmid DNA was done as described. Small-scale preparation of plasmid DNA from *E. coli* and yeast spheroplasts utilized an alkaline sodium dodecyl sulfate method (ref. 19, p. 90). Restriction endonuclease digestions and ligations with T4 DNA ligase were done as recommended by the suppliers. DNA restriction digests were analyzed by electrophoresis in 50 mM Tris/borate (pH 8.3) and 2 mM EDTA on 0.9% agarose gels.

Mitochondrial Preparation and Analysis. Mitochondrial and postmitochondrial supernatant (130,000 × g_{max}) fractions were prepared from yeast strain SEY2102 harboring

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Abbreviations: F_1-ATPase, the soluble mitochondrial ATPase (coupling factor 1) consisting of five nonidentical subunits—α, β, γ, δ, and ε in order of decreasing molecular weight; kDa, kilodalton(s); bp, base pair(s); kb, kilobase pair(s); X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; Ura⁺ and Ura⁻, uracil-independent and -dependent; Gly⁺ and Gly⁻, ability and inability to grow on glycerol.

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different plasmids as described (21). The cells were grown in 2× SD [2% glucose (dextrose)] supplemented with leucine at 40 mg/liter and histidine at 20 mg/liter. Density gradient analysis of washed mitochondria was performed on a linear 20–70% sucrose gradient containing 50 mM Tris·HCl at pH 7.4 and 0.5 mM phenylmethylsulfonyl fluoride. The gradients were spun 16 hr at 20,000 rpm in a Beckman SW 27.1 rotor. Published procedures were used to assay malate dehydrogenase (22), adenylate kinase (23), cytochrome *c* peroxidase (24), ATPase (25), $^{32}P_i$–ATP exchange (26), kynurinine hydroxylase (27), α-mannosidase (28), and NADPH–cytochrome *c* oxidoreductase (29). The following assays were performed as described but with the indicated changes: β-galactoside (ref. 17, p. 352) included 0.1% Triton X-100, and antimycin A-insensitive NADH–cytochrome *c* reductase (30) and cytochrome oxidase (31) assays contained antimycin A at 1 μg/ml. All reactions took place at 25°C. Protein concentrations were assayed by the Lowry method (32).

Immunoprecipitations and Electrophoresis Procedures. Yeast cells (1 OD_{600}/ml) were labeled in low-sulfate medium with $H_2{}^{35}SO_4$ (New England Nuclear, carrier-free) at 200 μCi/ml (1 Ci = 37 GBq) as described (33). Immunoprecipitation of labeled cells (34) with antisera directed against either β-galactosidase (gift from S. Dusing-Swartz) or the yeast F_1-ATPase β subunit (35) followed by electrophoresis on sodium dodecyl sulfate/7.5% polyacrylamide gels was done as described (36, 37).

RESULTS

Construction of the *ATP2–lacZ* Gene Fusion. pSEY101, an *E. coli*–yeast shuttle vector carrying a truncated *lacZ* gene, was used for construction of the *ATP2–lacZ* gene fusion. This plasmid contains selectable markers and origins for replication in both *E. coli* and yeast (Fig. 1). It also carries a *lacZ* gene that lacks all of its 5′ regulatory sequences as well as the coding sequences for the first 8 amino acids of the *lacZ* gene product, β-galactosidase. In their place, restriction enzyme sites for *Eco*RI, *Sma* I, and *Bam*HI are present (38).

The *ATP2* gene is contained within a 3.3-kb *Eco*RI fragment (15). This fragment carries approximately 1100 bp of DNA to the 5′ side and approximately 700 bp of DNA to the 3′ side of the *ATP2* structural gene. A *Bam*HI site is present within the *ATP2* gene at a site corresponding to amino acid position 350 in the *ATP2* gene product, the ATPase β subunit (479 amino acids total). DNA sequence analysis of the *ATP2* gene has shown that the reading frame across this *Bam*HI site is the same as the reading frame across the *Bam*HI site present in the *lacZ* fusion vector pSEY101 (15). It was, therefore, possible to construct an in-frame *ATP2–lacZ* gene fusion by transferring the 2.2-kb *Eco*RI/*Bam*HI *ATP2* DNA fragment carrying ≈1100 bp of DNA upstream of the β-subunit coding sequence as well as ≈1100 bp of the *ATP2* gene directly into pSEY101 (Fig. 1). When this construction, pβZ1, is used to transform either *E. coli* or yeast, β-galactosidase expression can be detected on plates with the chromogenic substrate X-Gal or by liquid assays. Plasmid pSEY101 does not give rise to detectable β-galactosidase activity in *E. coli* or yeast.

Expression of the *ATP2–lacZ* Gene Fusion in Yeast. Transformation of the *ura3* yeast strain SEY2102 with the pβZ1 plasmid yields uracil-independent (Ura^+) transformants that express β-galactosidase. These transformants are unstable in the absence of Ura^+ selection. Ura^- segregants, all of which have simultaneously lost detectable β-galactosidase activity, appear at a frequency of 30–50% per generation. This indicates that pβZ1 is maintained as a plasmid in these yeast cells.

The level of β-galactosidase expression directed by the *ATP2–lacZ* hybrid gene was determined in yeast cells after

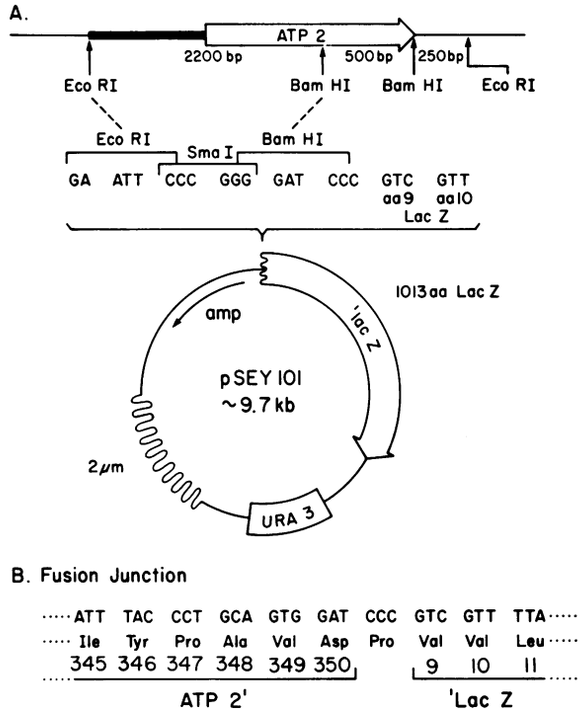

FIG. 1. Gene fusion between *ATP2* and *lacZ*. pSEY101 is a derivative of pCGS139 (gift of G. Vovis). The *Sma* I site normally present in the *URA3* region contained in this plasmid was removed by S1 nuclease treatment after digestion with *Xma* I. *Sal* I and *Xho* I sites at the 3′ side of *lacZ* in the vector pCGS139 were removed by digestion with both of these enzymes followed by religation. The 2.2-kilobase-pair (kb) *Eco*RI/*Bam*HI *ATP2* DNA was ligated into pSEY101 (*A*) as described in the text. This generates the uninterrupted open reading frame shown in *B*. bp, Base pairs; aa, amino acid.

growth in either glucose of galactose. Earlier studies have shown that the level of the mitochondrial F_1-ATPase subunits is glucose repressible (39). In addition, it has been observed (40) that the steady-state level of the F_1-ATPase β-subunit mRNA is increased 7-fold when cells are shifted from glucose to galactose as sole carbon source. After the cells had been shifted from glucose to galactose for 3 hr, the β-galactosidase activities measured for the *ATP2–lacZ* gene product increased from 127 to 500 units/mg of whole cell protein. These results indicate that expression of the *ATP2–lacZ* gene fusion on the plasmid pβZ1 follows the same pattern of regulation exhibited by the wild-type *ATP2* gene.

***ATP2–lacZ* Directs the Synthesis of a Hybrid Protein.** The DNA sequence data and the β-galactosidase expression studies described above for yeast cells harboring the pβZ1 plasmid all indicate that such cells are synthesizing a hybrid β-subunit–β-galactosidase protein. We tested for the presence of such a hybrid protein by first radioactively labeling SEY2102(pβZ1) cells with $^{35}SO_4^{2-}$ and then immunoprecipitating with antiserum directed against either β-galactosidase or the yeast F_1-ATPase β-subunit (Fig. 2). Both antisera specifically recognize a protein with an apparent molecular mass of 158 kDa on sodium dodecyl sulfate/polyacrylamide gels. Control cells containing a *CYC1–lacZ* gene fusion on plasmid pLG669-Z (8) show a single immunoprecipitable product of 120 kDa, which can be detected with the β-galactosidase antiserum but not with the β-subunit antiserum. The *CYC1* gene codes for the iso-1-cytochrome *c* protein, a mitochondrial inner membrane protein. The gene fusion on plasmid pLG669-Z contains ≈1100 bp of *CYC1* 5′ flanking DNA and the first codon of the *CYC1* coding sequence fused in frame to *lacZ*. This hybrid gene, therefore, directs the synthesis of essentially wild-type β-galactosidase, a normally

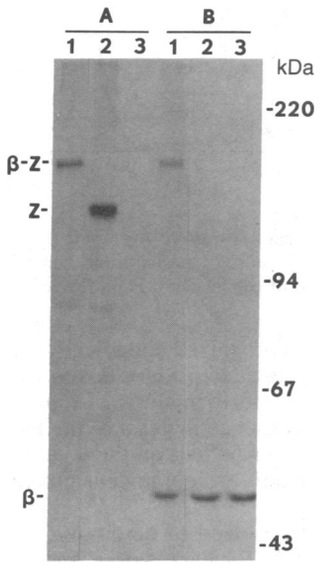

FIG. 2. Identification of the *ATP2–lacZ* hybrid protein in yeast. Yeast strain SEY2102 harboring the indicated plasmids was grown in midlogarithmic phase while Ura^+ selection was maintained, then labeled with $^{35}SO_4^{2-}$ and processed for immunoprecipitation. In each case, the labeled cell homogenates were divided in half and immunoprecipitated with antiserum to either β-galactosidase (*A*) or F_1-ATPase β subunit (*B*). The labeled cell homogenates were prepared from SEY2102 containing the following plasmids: lanes 1, pβZ1; lanes 2, pLG669-Z, and lanes 3, pSEY101. The molecular mass standards used were ferritin (220 kDa), phosphorylase b (94 kDa), bovine serum albumin (67 kDa), and ovalbumin (43 kDa). β, β subunit; Z, β-galactosidase.

Table 1. Expression and localization of β-galactosidase fusion proteins

Fusion plasmid	β-Galactosidase level: Whole cell, units/mg	Cell fractions: Pellet, total units	Cell fractions: Supernatant, total units	Activity pelleted, % of total
pβZ1	127	19,261	944	95.3
pLG669-Z	993	1,094	101,132	1.1

Analysis of the β-galactosidase distribution in cell fractions was performed on cells grown to 2×10^7 cells per ml in 2× SD medium maintaining Ura^+ selection. An identical wet weight of cells was converted to spheroplasts and disrupted for preparation of mitochondria. After removal of cell debris at 3000 × *g* for 3 min, the supernatant was respun at 9000 × *g* for 10 min. The 9000 × *g* pellet was washed once. The supernatants were combined and the pellet was resuspended in 0.6 M mannitol/50 mM Tris·HCl, pH 7.4/0.5 mM phenylmethylsulfonyl fluoride. Mitochondria prepared in this manner contain the following levels of whole cell marker enzyme activities: NADPH–cytochrome reductase, 15%; α-mannosidase, 20%; cytochrome oxidase, 90%; and vanadate-sensitive ATPase, 14%. (One unit is the amount of enzyme that hydrolyzes 1 nmol of *o*-nitrophenyl β-D-galactoside per min at 28°C.)

soluble cytoplasmic enzyme. The above results demonstrate that the *ATP2–lacZ* gene fusion does direct the synthesis of a hybrid protein consisting of both β-subunit and β-galactosidase polypeptide sequences.

DNA sequence analysis of the *ATP2* gene indicates that, in the *ATP2–lacZ* gene fusion, coding sequences for the first 350 amino acids (≈37.5 kDa) of the β-subunit protein should be present. Wild-type β-galactosidase has a mass of 116.4 kDa (41). Therefore, the *ATP2–lacZ* gene fusion described here is expected to encode a hybrid protein of approximately 154 kDa. The molecular mass observed above for the β-subunit–β-galactosidase hybrid protein agrees well with that predicted by the DNA sequence data. Also, as expected from previous studies (8), the *CYC1–lacZ* gene fusion on plasmid pLG669-Z directs the synthesis of a hybrid protein with an apparent molecular mass that is very similar to that of wild-type β-galactosidase.

Cellular Location of the *ATP2–lacZ* Hybrid Gene Product. The location in yeast cells of β-galactosidase activity expressed from the *ATP2–lacZ* gene fusion was determined. This enzyme activity provides an indicator of the distribution of the β-subunit–β-galactosidase hybrid protein in these cells. As a control in this analysis, β-galactosidase activity expressed from the plasmid carrying the *CYC1–lacZ* gene fusion, pLG669-Z, also was monitored.

To compare the subcellular distribution of β-galactosidase activity in each of the above cases, whole cell homogenates were prepared from equal amounts of the yeast strains SEY2102(pβZ1) and SEY2102(pLG669-Z) and resolved into mitochondria-enriched 9000 × *g* pellet and supernatant fractions. The amount of β-galactosidase activity detected in the whole cell homogenates corresponds well with the total activity present in the intact cells. The 9000 × *g* pellet (enriched in mitochondria) contains greater than 95% of the β-galactosidase activity expressed from the *ATP2–lacZ* gene fusion and less than 5% of the *CYC1–lacZ*-encoded enzyme activity (Table 1). Moreover, immunoprecipitation analysis has shown that it is the intact β-subunit–β-galactosidase hybrid protein that is found in the mitochondria-enriched fraction (see Fig. 2). However, when the mitochondrial fraction was treated with Triton X-100 under conditions that solubilize the wild-type ATPase and cytochrome oxidase activities, it was found that >90% of the β-galactosidase activity is not solubilized. This apparent insolubility of the hybrid protein could lead to its fortuitous cofractionation with mitochondria in the 9000 × *g* pellet. To avoid this ambiguity, crude mitochondria were also fractionated on sucrose and Percoll density gradients in which mitochondria do not pellet. Under these conditions, the β-galactosidase activity continues to cofractionate with the mitochondrial marker enzyme cytochrome oxidase and not with contaminating cellular organelle marker enzymes (Fig. 3). These results indicate that the *ATP2* sequences present in the *ATP2–lacZ* gene fusion contain sufficient information to direct the hybrid protein to mitochondria. The *CYC1–lacZ* gene fusion appears to lack this information.

Upon import into mitochondria, the wild-type β-subunit protein becomes inaccessible to externally added proteinase K *in vitro* (4). We have found that mitochondrial β-galactosidase activity expressed from the *ATP2–lacZ* hybrid gene exhibits a similar proteinase K-resistant character (80% of the activity is protected). However, greater than 95% of the β-galactosidase activity as well as cytochrome oxidase is inactivated by protease when the mitochondria are solubilized with 0.5% Triton X-100. The results suggest that the β-subunit–β-galactosidase hybrid protein is protected by at least one membrane protease barrier in the isolated mitochondria. Further tests will be required to determine the precise disposition of the hybrid protein within the organelle.

Unusual Phenotype Exhibited by Yeast Cells Harboring the pβZ1 Plasmid. In addition to directing the synthesis of a hybrid protein, the *ATP2–lacZ* gene fusion present on plasmid pβZ1 prevents the growth of otherwise wild-type yeast cells on a nonfermentable carbon source (e.g., glycerol). This respiration-negative phenotype (Gly^-) appears to result from mitochondrial import of the β-subunit–β-galactosidase hybrid protein. Simple expression of soluble β-galactosidase from another hybrid gene, *CYC1–lacZ*, does not lead to this phenotype. Also, the presence in yeast of the wild-type *ATP2* gene clone or of a truncated derivative of this clone lacking *ATP2* sequences 3′ of the *Bam*HI site (15) does not confer this growth property. Finally, this phenotype does not appear to result simply from the presence of glycerol in the growth medium since cells containing the pβZ1 plasmid grow normally in media containing both glucose and glycerol. It seems most likely that the Gly^- phenotype results from some aspect of the derepressed *ATP2–lacZ* hybrid gene product's import into or location within mitochondria.

If the *ATP2–lacZ*-encoded hybrid protein becomes jammed during entry into the mitochondrial matrix, it might be expected to interfere with either the import or the function of other mitochondrial proteins. To examine this, we

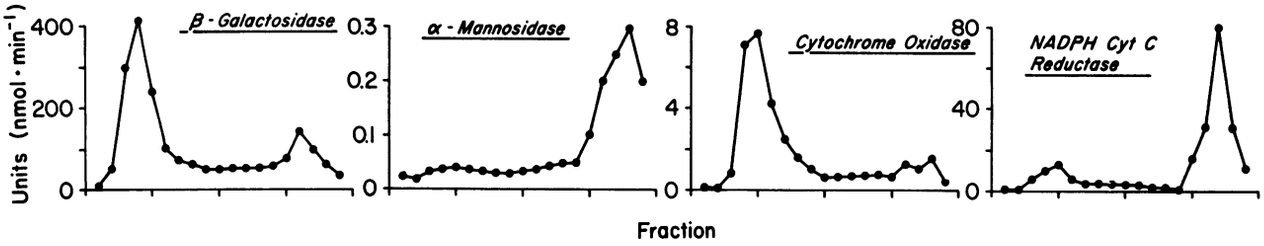

FIG. 3. Cytochrome oxidase and β-galactosidase cofractionate on a sucrose density gradient. Fresh mitochondria were prepared from SEY2102(pβZ1) as described for Table 1. The mitochondria (3.8 mg of protein) were layered on a linear 20–70% sucrose gradient and centrifuged. Nineteen fractions (1 ml each) were collected and assayed for the indicated marker enzymes. The bottom of the gradient is on the left.

have assayed selected mitochondrial matrix (malate dehydrogenase), inner membrane (cytochrome *c* oxidase and oligomycin-sensitive ATPase), outer membrane (antimycin-insensitive NADH–cytochrome *c* reductase and kynurenine hydroxylase), and intermembrane space (cytochrome *c* peroxidase and adenylate kinase) marker enzyme activities in cells harboring either the pβZ1 or the pLG669-Z plasmid. We found that the *ATP2–lacZ* gene fusion did not cause any significant change in the levels of activity and presumably the import of these marker enzymes (data not shown). It is important to note, however, that these analyses were done with mitochondria isolated from cells grown on a carbon source (glucose) that does not require a functional organelle.

The oligomycin-sensitive ATP-cleaving activity of the F_1-ATPase appears to be unaffected by the presence of the *ATP2–lacZ* gene fusion (see above). This enzyme also functions in ATP synthesis, which can be measured by determining the $^{32}P_i$–ATP exchange activity of the enzyme. When this activity was measured, it was found that mitochondria harboring the β-subunit–β-galactosidase hybrid protein have 1/10th the $^{32}P_i$–ATP exchange activity (1.15 nmol/min·mg) of mitochondria lacking the hybrid protein (12.28 nmol/min·mg). The marked decrease in the level of the ATP-synthesizing capacity of this energy-transducing enzyme may contribute to the failure of yeast strains carrying the pβZ1 plasmid to grow on a nonfermentable carbon source.

The *ATP2–lacZ*-dependent Gly^- phenotype could prove to be a useful genetic handle for extending these studies on mitochondrial protein import. We have found that it is possible to isolate Gly^+ mutant yeast cells carrying the pβZ1 plasmid. Most of the Gly^+ mutants no longer express any detectable β-galactosidase activity, further suggesting that it is the β-subunit–β-galactosidase hybrid protein that leads to this Gly^- phenotype. Three Gly^+ mutants, however, have been isolated that still express active β-galactosidase. Determination of the precise effects and nature of these mutations and others should provide us with additional insights into the molecular events of protein targeting to and localization within the yeast mitochondrion.

DISCUSSION

The gene encoding the mitochondrial F_1-ATPase β-subunit (*ATP2*) of *S. cerevisiae* has been fused to the *lacZ* gene of *E. coli*. A variety of properties relating to the expression and localization of the hybrid gene product have been analyzed.

DNA sequence information predicts that the hybrid protein coded for by the *ATP2–lacZ* gene fusion consists of 350 amino acids of the β-subunit protein at its amino terminus and a large active fragment (1013 amino acids) of β-galactosidase at its carboxyl terminus. Expression in yeast of β-galactosidase from the hybrid gene is regulated in a manner similar to that observed for the wild-type *ATP2* gene; repression in glucose medium and derepression by growth in the absence of glucose. Also, as predicted, we have shown that the *ATP2–lacZ* gene fusion directs the synthesis of a hybrid protein of ≈154 kDa that can be recognized specifically by antiserum directed against either β-galactosidase or the F_1-ATPase β-subunit protein. Furthermore, analysis of immunoprecipitates indicates that the presence in yeast of the hybrid *ATP2–lacZ* gene has no apparent effect on the level of expression of the wild-type β-subunit protein in cells grown on glucose (Fig. 2).

β-Galactosidase activity has been used to determine the cellular location of the β-subunit–β-galactosidase hybrid protein. We have found that >90% of the β-galactosidase activity in yeast cells carrying the *ATP2–lacZ* gene fusion on the plasmid pβZ1 cofractionates with mitochondria. In addition, protease protection studies indicate that the hybrid β-subunit–β-galactosidase protein is being delivered through at least the outer membrane protease barrier of mitochondria. A control gene fusion, *CYC1–lacZ* (8), between the mitochondrial iso-1-cytochrome *c* gene and *lacZ* directs the synthesis of soluble cytoplasmic β-galactosidase activity. This gene fusion contains only the 5′ regulatory sequences and the initiating methionine codon of *CYC1* fused to *lacZ*. Thus, it appears that sequences upstream of the structural gene do not direct mitochondrial import. This is supported by the fact that many mitochondrial proteins, including the ATPase β subunit, have been shown *in vitro* to be capable of being imported into mitochondria posttranslationally (4). Our results indicate that the information necessary for targeting the β-subunit protein to the mitochondrion resides in an amino-terminal portion of the polypeptide. The β-subunit protein, like several other proteins destined for import into the mitochondrion, is made initially as a precursor with an amino-terminal extension of some 15–20 amino acid residues (4). This pre sequence is proteolytically removed during import by a soluble protease present within the mitochondrial matrix (12, 13, 42). It has been proposed that the pre sequence may function to bind the polypeptide specifically to the mitochondrial import machinery, thereby permitting initiation of translocation of the protein into the organelle. Our present data concerning the *ATP2* gene product are consistent with such a model. Carboxyl-terminal amino acids do not appear to be necessary for directing mitochondrial protein import. The *ATP2–lacZ* gene fusion is missing the coding sequences for 129 carboxyl-terminal amino acid residues of the β-subunit protein. In addition, putting a large heterologous polypeptide such as β-galactosidase in place of these amino acids appears not to block at least initiation of import of the hybrid polypeptide. Isolation of additional size classes of *ATP2–lacZ* gene fusions should allow us to define in greater detail the limits of *ATP2* information required for initial binding and for import of the β-subunit protein.

Another possible approach we can employ to define information responsible for directing import of the ATPase β-subunit protein takes advantage of an interesting phenotype exhibited by cells containing the *ATP2–lacZ* gene fusion. Yeast cells containing the pβZ1 plasmid are respiration negative. These cells cannot grow on a nonfermentable carbon source such as glycerol. Several lines of both biochemical and ge-

netic evidence support the contention that this phenotype results from localization of the β-subunit–β-galactosidase hybrid protein in mitochondria.

(*i*) Simple expression of high levels of cytoplasmic β-galactosidase from the *CYC1–lacZ* gene fusion does not lead to this phenotype.

(*ii*) The Gly^- phenotype is not the result of having *ATP2* sequences present in multiple copies or the overproduction of the β-subunit protein since a plasmid similar to pβZ1 carrying either the wild-type *ATP2* gene or an *ATP2* gene lacking some of its 3′ coding sequence is Gly^+.

(*iii*) Gly^+ mutants isolated from yeast strains harboring the pβZ1 plasmid are most often negative for β-galactosidase expression, suggesting that a block in synthesis of the hybrid *ATP2–lacZ* gene product overcomes the Gly^- phenotype.

(*iv*) The enzyme activities and presumably the import of most of the mitochondrial enzymes tested appears normal in yeast cells carrying the pβZ1 plasmid, at least when these cells are grown on glucose, a repressing carbon source. Even under these growth conditions, however, a significant decrease was observed in the function of the ATPase energy-transducing complex (uncoupler-sensitive $^{32}P_i$–ATP exchange). This effect may contribute to the respiration-negative phenotype observed.

The present data are consistent with at least two possible models. The β-subunit–β-galactosidase hybrid protein may be delivered into the mitochondrial matrix and in this location be able to affect the assembly or functioning of the wild-type ATPase complex so as to confer an ATPase-negative phenotype (Gly^-) to cells. Alternatively, the hybrid protein may become jammed at a terminal step during its transit into mitochondria. The hybrid protein thereby could block the import or functioning of other essential mitochondrial proteins and thus give rise to the respiration-defective phenotype. A set of conditional-lethal phenotypes similar to those observed here for the *ATP2–lacZ* gene fusion has been seen previously in *E. coli* strains containing fusions between secretory proteins and β-galactosidase (43). In these instances the conditional-lethal phenotype was exploited successfully to obtain large numbers of mutations in the sequences that direct the secretion of these proteins (5). Mutations in the export machinery of *E. coli* have also been defined by this approach (6, 44). It seems likely that a similar approach could be employed here, in yeast, with the *ATP2–lacZ* gene fusion by isolating Gly^+ mutants that still express β-galactosidase activity. We have already isolated three such mutants. It is hoped that these mutations will aid us in defining the sequences and cellular machinery that function in the efficient delivery of mitochondrial proteins to their final destinations.

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